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Comparison of Spontaneous Antibiotic Resistance Frequency of *Salmonella* Typhimurium Growth in Glucose Amended Continuous Culture at Slow and Fast Dilution Rates

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The objective of the study was to determine the frequency of spontaneous acquisition of resistance to select antibiotics by *Salmonella* Typhimurium (ST) when grown in glucose amended continuous flow culture at slow ($D = 0.025\ h^{-1}$) or fast ($D = 0.27\ h^{-1}$) dilution rates. The bacterium was grown in LB minimal medium (pH 6.25) containing no antibiotics. Upon achieving steady state, samples were plated to tryptic soy agar (TSA) alone or supplemented (per ml) with 2 and 16 μg oxytetracycline, 4 and 16 μg tetracycline, 2 and 64 μg kanamycin, and 0.25 and 2 μg enrofloxacin. Regardless of growth rate, CFU of resistant ST from the TSA containing antibiotics was less than 2×10^1 except for 2 μg kanamycin and 0.25 μg enrofloxacin treatments (higher than 1×10^9 and 4×10^7 CFU of resistant ST for trials 1 and 2, respectively). Frequency of recovering resistant ST from the TSA containing the higher antibiotic concentrations was less than 1 in 10^9 for all antibiotics, but was higher on the media containing 2 μg kanamycin and 0.25 μg enrofloxacin at both slow and fast growth rates. In general, minimal susceptibility differences were detected for isolates from slow and fast dilution rates.

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Key Words: Antibiotic resistance; Dilution rate; Continuous culture; *Salmonella* Typhimurium.

INTRODUCTION

Bacterial resistance to an increasing number of antimicrobial drugs is becoming a serious human health issue because increased drug resistance of pathogenic bacteria reduces the efficacy of antimicrobial treatment.[1] Foodborne *Salmonella* spp. are well-known human pathogens, and strains of *Salmonella* resistant against various antimicrobial agents have become a major public health concern.[2] Development of antibiotic-resistant *Salmonella* strains continues to increase in the frequency of *Salmonella* infections.[3] Although *Salmonella* is found in many sources, the intestinal tract of animals is the primary reservoir, and intensive animal production can be favorable environments for long-term establishment of *Salmonella*.[4] The common use of antimicrobial-agents in human and veterinary medicine and animal production for growth promotion has caused the increase in the cases of antimicrobial-resistant pathogens.[5,6] This may be dependent on exposure to multiple antibiotics over extended periods of time. Guerra et al.[5] indicated that 333 *Salmonella* strains tested were susceptible to amikacin, ceftazidime, ciprofloxacin, and imipenem, and 31% were susceptible to all antimicrobials tested. The most frequent types of resistance were to sulfadiazine, tetracycline, streptomycin, spectinomycin, ampicillin, and chloramphenicol. They also observed multidrug resistance pattern of *Salmonella*.

The occurrence of multiresistant *Salmonella* infections has increased in the recent year.[7] *Salmonella* Typhimurium shows high frequency (52%) of resistance to multiple antibiotics among *Salmonella* serotypes.[2] Since growth conditions can vary considerably in the environments that *Salmonella* spp. can be found, it is important to evaluate the frequency of antibiotic resistance as a function of growth conditions. There is a little information on influences of growth rates on frequency and level of antibiotic resistance. Continuous culture enables the evaluation of external factors on growth kinetics by controlling growth rate as a function of dilution rate. Thus, the objective of the study was to determine the frequency of spontaneous acquisition of resistance to selected antibiotics by *Salmonella* Typhimurium when grown in glucose limited continuous flow culture at slow or fast dilution rates.

MATERIALS AND METHODS

Preparation of *Salmonella* Typhimurium Inoculum

The *Salmonella* Typhimurium inoculum was taken from a frozen glycerol stock, thawed and streaked onto an LB agar. Plates were incubated overnight

at 37°C. After incubation a colony was used to inoculate 3 ml of LB broth in a 15 ml borosilicate tube. The tube was incubated at 37°C for 18 h (OD of 0.6, A_{600}). Inoculum was thoroughly agitated and 1 ml was used to inoculate each of two continuous flow culture systems.

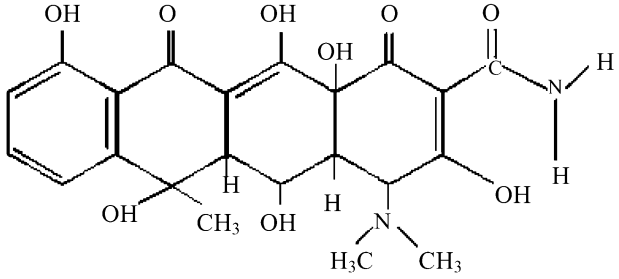
Establishment of Apparatus and Continuous Flow Culture Systems

Two continuous flow culture systems (CFC) were established in BioFlo chemostat (New Brunswick Scientific Company, Edison, NJ) with a total vessel volume of 1.0 liter. Chemostats were prepared according to *New Brunswick Scientific Company Handbook*. LB broth medium (described above) and chemostats were sterilized (40 min at 21 psi pressure and at 121°C), with a 500 ml medium volume each. Chemostats were maintained anaerobically (CO_2) at 39°C and agitated at 100 rpm with a 98% turnover rate. Two chemostats were operated concurrently to facilitate changes in dilution rates (D) and the resulting responses combined for data analyses. All samples taken in these chemostats were conducted at steady state. Chemostats were adjusted to two different dilution rates: 0.0125 h^{-1} (defined as slow growth rate) and 0.27 h^{-1} (defined as fast growth rate).

Evaluating Antibiotic Resistance Frequency of *Salmonella* Typhimurium

Salmonella Typhimurium was grown in glucose limited continuous flow culture at slow ($D = 0.025\ h^{-1}$) or fast ($D = 0.27\ h^{-1}$) dilution rates. The bacterium was grown in LB minimal medium (pH 6.25) containing no antibiotics. Upon achieving steady state, samples were plated to tryptic soy agar (TSA) alone or supplemented (per ml) with 2 and 16 μg oxytetracycline (Fig. 1: 4-Dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide), 4 and 16 μg tetracycline (Fig. 1: 4-Dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide), 2 and 64 μg kanamycin (Fig. 2: O-3-Amino-3-deoxy-alpha-D-glucopyranosyl-(1-6)-O-[6-amino-6-deoxy-alpha-D-glucopyranosyl-(1-4)]-2-deoxy-D-streptamine) and 0.25 and 2 μg enrofloxacin (Fig. 2: 3-Quinolinecarboxylic acid, 1,4-dihydro-1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-4-oxo-1,4-Dihydro-1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-4-oxo-3-quinolinecarboxylic acid). After 24 h incubation at 37°C, *Salmonella* colonies from each treatment were counted, and representative isolates from the antibiotic supplemented TSA for their susceptibility to these respective antibiotics were evaluated using the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, OH,

Oxytetracycline



Tetracycline

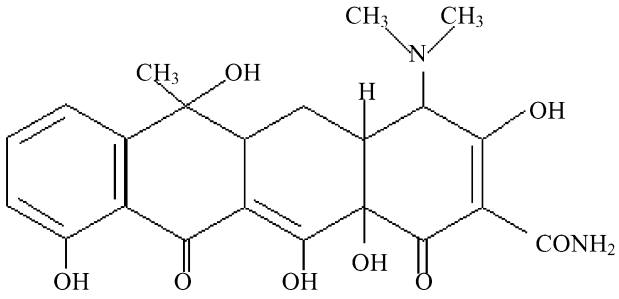


Figure 1: The chemical structures of oxytetracycline and tetracycline.

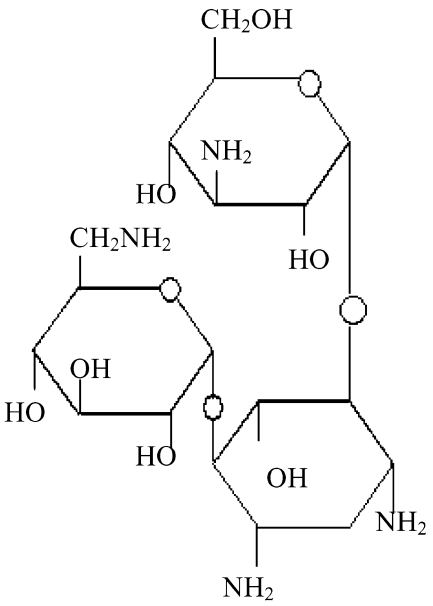
USA). Minimum inhibitory concentrations for oxytetracycline, tetracycline, kanamycin, and enrofloxacin were determined by the National Committee for Clinical Laboratory Standards.[8] Resistance breakpoints were determined using NCCLS standards.[8]

RESULTS AND DISCUSSION

Salmonella Typhimurium Plate Counts

Plate counts of *Salmonella* Typhimurium (ST) on the media with and without antibiotics of trial 1 are presented in Table 1. After 24 h incubation at 37°C, recovery of ST from unsupplemented TSA was 3.0 and 2.6 $\times$ 10^9 CFU for slow and fast growing cultures, respectively. CFU of resistant ST from the TSA containing 0.2 and 16 μg oxytetracycline, 16 μg tetracycline, 64 μg kanamycin, and 2 μg enrofloxacin was less than 2 $\times$ 10^1. However, CFU of resistant ST from TSA containing 2 μg kanamycin and 0.25 μg enrofloxacin was relatively high (CFU $>$ 1 $\times$ 10^9) at both growth rates. The 4 μg tetracycline treatment at the fast growth rate (6 $\times$ 10^3) yielded a higher CFU population

Kanamycin



Enrofloxacin

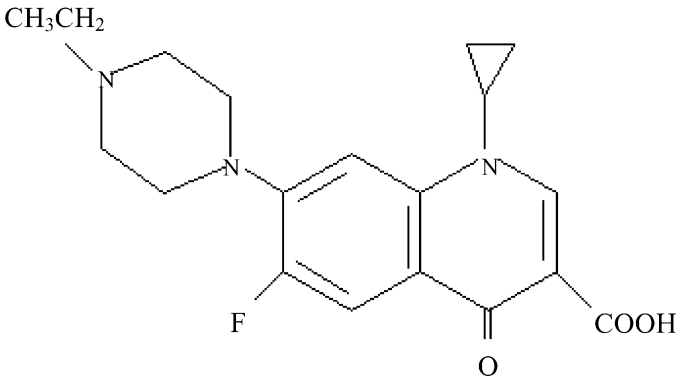


Figure 2: The chemical structure of kanamycin and enrofloxacin.

of resistant ST compared to the same treatment at a slow growth rate (1×10^0).

Plate counts of ST on the media with and without antibiotics of trial 2 are shown in Table 2. The recovery trends of resistant ST were similar to the trial 1. Regardless of growth rate, CFU of resistant ST from the TSA containing antibiotics was less than 1×10^1 except for 2 μg kanamycin and 0.25 μg enrofloxacin treatments. Medium containing 2 μg kanamycin had 6.3×10^7 and 9.1×10^8 CFU of resistant ST at fast and slow growth rates, respectively.

Table 1: Plate counts of *Salmonella* Typhimurium on the media with and without antibiotics (trial 1).

Antibiotic	Slow growth rate ($D^1 = 0.025$)	Fast growth rate ($D = 0.27$)
No antibiotics	3.0×10^9	2.6×10^9
0.2 μg oxytetracycline	2.0×10^1	1.0×10^0
16 μg oxytetracycline	1.0×10^1	1.0×10^0
4 μg tetracycline	1.0×10^1	6.0×10^3
16 μg tetracycline	1.0×10^1	1.0×10^0
2 μg kanamycin	5.0×10^9	4.4×10^9
64 μg kanamycin	1.0×10^1	1.0×10^0
0.25 μg enrofloxacin	3.5×10^9	1.8×10^9
2 μg enrofloxacin	1.0×10^1	1.0×10^0

[1]Dilution rate.

Frequency of Antibiotic Resistant *Salmonella* Typhimurium

Effect of growth rate on frequency of recovering ST resistant to select antibiotics for trials 1 and 2 is shown in Tables 3 and 4, respectively. Frequency of recovering resistant ST from TSA containing 4 μg tetracycline at the fast growth rate (2.3×10^{-6}) was higher than at the slow growth rate (3.3×10^{-9}) in trial 1. Relative difference between fast and slow growth rates was 697. However, in trial 2, the relative difference was reduced to 2.8. Frequency of recovering resistant ST from the TSA containing the higher antibiotic concentrations was less than 1 in 10^9 for all antibiotics tested. Frequency of recovering resistant ST was higher on the media containing 2 μg kanamycin and 0.25 μg enrofloxacin at both slow and fast growth rates. Relative differences in frequency of recovering survivors between fast and slow growth rates were 40 and 35 on the media containing 2 μg kanamycin and 0.25 μg enrofloxacin, respectively. Kanamycin is one of the aminoglycoside antibiotics that has two glycosides joined to an

Table 2: Plate counts of *Salmonella* Typhimurium on the media with and without antibiotics (trial 2).

Antibiotic	Slow growth rate ($D^1 = 0.025$)	Fast growth rate ($D = 0.27$)
No antibiotics	1.5×10^9	5.2×10^8
0.2 μg oxytetracycline	1.0×10^1	1.0×10^0
16 μg oytetracycline	1.0×10^0	1.0×10^0
4 μg tetracycline	1.0×10^0	1.0×10^0
16 μg tetracycline	1.0×10^0	1.0×10^0
2 μg kanamycin	6.3×10^7	9.1×10^8
64 μg kanamycin	1.0×10^0	1.0×10^0
0.25 μg enrofloxacin	4.6×10^7	5.7×10^8
2 μg enrofloxacin	1.0×10^1	1.0×10^0

[1]Dilution rate.

Table 3: Effect of growth rate on frequency of recovering *Salmonella* Typhimurium resistant to select antibiotics (trial 1).

Antibiotic	Frequency of recovering survivors (D = 0.025)	Frequency of recovering survivors (D = 0.27)	Relative difference
0.2 μg oxytetracycline	6.7×10^{-9}	3.9×10^{-10}	−17X
16 μg oxytetracycline*	3.3×10^{-9}	3.9×10^{-10}	−8.5X
4 μg tetracycline	3.3×10^{-9}	2.3×10^{-6}	+697X
16 μg tetracycline*	3.3×10^{-9}	3.9×10^{-10}	−8.5X
2 μg kanamycin	1.7×10^{0}	1.7×10^{0}	1X
64 μg kanamycin*	3.3×10^{-9}	3.9×10^{-10}	−8.5X
0.25 μg enrofloxacin	1.2×10^{0}	6.9×10^{-1}	−1.7X
2 μg enrofloxacin*	3.3×10^{-9}	3.9×10^{-10}	−8.5X

*Resistance breakpoints.

aminocyclitol ring, and contains replacements of key functional groups that are susceptible to structural deactivation by bacterial enzymes.[9] However, tetracycline and oxytetracycline are not structurally modified by bacterial enzymes. Normally, bacteria obtain their resistance to the tetracyclines through the drug efflux mechanism by which the drug is pumped out from the cell.[9,10]

Consistent patterns of resistance have been observed for several of these antibiotics and have also been observed in food animal production environments. Delsol, Woodward, and Roe[11] evaluated the effect of a single five-day enrofloxacin treatment on *Salmonella* Typhimurium DT104 in a pig model. Their results indicated that *Salmonella* counts were 100-fold higher in enrofloxacin treatment pigs inoculated with cyclohexane- and nalidixic acid-resistant ST for two weeks post-treatment than the untreated pigs. Antunes et al.[4] reported that a total of 75% of *Salmonella* isolates from poultry products were resistant to one or more of the antimicrobial agents tested. Fifty percent of *Salmonella*

Table 4: Effect of growth rate on frequency of recovering *Salmonella* Typhimurium resistant to select antibiotics (trial 2).

Antibiotic	Frequency of recovering survivors (D = 0.025)	Frequency of recovering survivors (D = 0.27)	Relative difference
0.2 μg oxytetracycline	6.7×10^{-9}	1.9×10^{-9}	−3.5X
16 μg oxytetracycline*	6.7×10^{-10}	1.9×10^{-9}	2.8X
4 μg tetracycline	6.7×10^{-10}	1.9×10^{-9}	2.8X
16 μg tetracycline*	6.7×10^{-10}	1.9×10^{-9}	2.8X
2 μg kanamycin	4.2×10^{-2}	1.7×10^{0}	40X
64 μg kanamycin*	6.7×10^{-10}	1.9×10^{-9}	2.8X
0.25 μg enrofloxacin	3.1×10^{-2}	1.1×10^{0}	35X
2 μg enrofloxacin*	6.7×10^{-9}	1.9×10^{-9}	2.8X

*Resistance breakpoints.

Table 5: Effect of growth rate on susceptibility of *Salmonella* Typhimurium to select antibiotics.

	MIC[1] of survivor from slow growth rate ($D^2 = 0.025$)				MIC of survivor from fast growth rate ($D = 0.27$)			
Antibiotic challenge	**Oxytetracycline**	**Tetracycline**	**Kanamycin**	**Enrofloxacin**	**Oxytetracycline**	**Tetracycline**	**Kanamycin**	**Enrofloxacin**
				(μg)				
0.2 μg oxytetracycline	8	34	4	1	4	32	8	1
16 μg oxytetracycline*	4	32	4	0.5	4	32	16	1
4 μg tetracycline	16	64	2	2	8	64	8	4
16 μg tetracycline*	4	32	2	1	NA[3]	NA	NA	NA
2 μg kanamycin	8	32	8	0.5	4	32	16	1
64 μg kanamycin*	NA	NA	NA	NA	NA	NA	NA	NA
0.25 μg enrofloxacin	NA	NA	NA	NA	4	32	8	1
2 μg enrofloxacin*	NA	NA	NA	NA	4	64	16	4

*Resistance breakpoints.
[1]Minimum inhibition concentration.
[2]Dilution rate.
[3]Not available.

were resistant to enrofloxacin, and the frequency of *Salmonella* resistant to streptomycin, tetracycline, kanamycin was 39, 36, and 3%, respectively. Several studies have reported that one of the most common resistances in *Salmonella* isolates was against tetracycline (approximately 36%) in poultry products.[12,13]

Poultry products are common sources in the transmission of *Salmonella* and are potentially higher sources of infection than other animal products.[14] In the present study, we evaluated the effect of growth rate on susceptibility of ST to select antibiotics using NCCLS (Table 5). The result suggests that there were little difference in susceptibilities of ST recovered at the slow and fast growth rates. This indicates that growth physiology extremes as least as a function of slow versus fast growth rate has minimal influence on spontaneous appearance and patterns of antibiotic resistance in this strain of *Salmonella*. Future studies are needed to examine the influence of dilution rate on the genetic transfer frequency between antibiotic resistant and susceptible strains of *Salmonella* when incubated together in the same continuous culture growth chamber.

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